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*MECHANISM OF ACTION OF PENICILLINS: A PROPOSAL BASED ON THEIR STRUCTURAL SIMILARITY TO ACYL-D-ALANYL-D-ALANINE**

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The mechanism by which penicillins kill bacterial cells has been of interest since their discovery in 1929.[1] In retrospect, three important observations stand out from the early studies. In general, penicillins kill gram-positive bacteria more effectively than gram-negative bacteria.[2] They kill rapidly dividing cells but not resting cells,[3] and induce morphological alterations in treated cultures.[4, 5] As early as 1946 it was suggested that the formation of bizarre and filamentous forms could be accounted for by the loss of integrity of the cell wall.[5]

Subsequently, cell walls were isolated and examined.[6] The walls of gram-positive bacteria have a remarkably simple composition compared to those of gram-negative organisms, a major difference between the two groups being the occurrence of lipid in the latter. Lysozyme[7] is a glycosidase which kills sensitive bacteria by solubilizing the cell wall.[8] In hypertonic sucrose broths, sensitive bacilli are not lysed but are converted to spherical forms termed protoplasts.[9] Thus, the wall determines the shape of bacteria and is responsible for their stability.

These observations set the stage for the hypothesis that penicillin (N-acyl 6-aminopenicillanic acid) is a highly specific inhibitor of bacterial cell wall synthesis.[10-13] This hypothesis was based both on formation of penicillin-induced spheroplasts of *E. coli* and on the accumulation of a uridine nucleotide in penicillin-treated *S. aureus* which, because of its structural similarity to the cell wall, appeared to be a biosynthetic precursor of the cell wall. This hypothesis has been amply confirmed by direct isotopic measurements of cell wall synthesis carried out with several penicillins and in both gram-negative and gram-positive bacteria (e.g., ref. 14).

The exact mechanism by which penicillin inhibits cell wall synthesis has been elusive. A linear cell wall glycopeptide is synthesized from uridine diphosphoacetylmuramyl · L-ala · D-glu · L-lys · D-ala · D-ala (UDP-MurNAc-pentapeptide), uridine diphosphoacetylglucosamine (UDP-GlcNAc), and other required substrates.[15-19] It is now generally agreed, despite one report that this polymerization reaction is absent from particles prepared from cells pretreated with penicillin,[15] that all of the reactions which lead to the synthesis of the linear glycopeptide are insensitive to penicillin, whether the antibiotic is added to the enzyme or to cells prior to preparation of the enzyme.[16, 17] The natural glycopeptide is crosslinked by peptide bridges, and Martin, from studies of the structure of the cell wall glycopeptide in normal cells and in penicillin-induced L-forms of *Proteus mirabilis*, has suggested that penicillin blocks formation of these peptide crosslinks.[20, 21]

Consideration of the results of recent studies of cell wall structure and biosynthesis in *S. aureus* and other bacteria suggests that the terminal reaction in cell wall synthesis may be a transpeptidation in which linear glycopeptides are crosslinked to form a three-dimensional network. Based on molecular models, it is proposed that penicillin is a structural analog of acyl-D-alanyl-D-alanine in the linear glycopeptide, and inhibits transpeptidation by reacting preferentially with the enzyme binding site for this substrate. In support of this thesis it has been shown that in the presence of penicillin the proportion of uncrosslinked disaccharide-peptide units incorporated into the cell wall glycopeptide is greatly increased, and that these nascent units retain their carboxyl terminal D-alanine. This proposal differs in important details from a similar hypothesis recently proposed by Wise and Park.[22]

Structure of the Cell Wall Glycopeptide.—The glycopeptide in all species examined contains alternating acetylglucosamine and acetylmuramyl-peptide fragments. The idea that glycopeptide units are crosslinked through their peptide moieties was first derived from the paucity of free amino groups in cell walls which indicated that both amino groups of the dibasic amino acids (e.g., L-lysine or α,ϵ-mesodiaminopimelic acid) were substituted to form a branched peptide.[23-25] Also analyses of small fragments obtained from lysozyme digests of cell walls of several species showed that peptide-linked dimers could be isolated[26, 27] in small yield. One apparent anomaly was consistently reported in analyses of cell walls; they contained two alanine residues per glutamic acid residue although UDP-MurNAc-pentapeptide, the precursor of this structure, contained three alanine residues. It could not be determined whether these analyses were the statistical average of MurNAc-tripeptides containing one alanine and MurNAc-pentapeptides containing three alanines, which was possible from biosynthetic considerations, or whether the wall contained tetrapeptides. However, recent degradation of the cell wall of *S. aureus* with hydrolytic enzymes has shown that 90 per cent of the peptide units are tetra-

peptides, thus showing conclusively that one D-alanine had been lost from the pentapeptide in the course of wall synthesis.[28, 29] The other 10 per cent of the peptide units appeared to be pentapeptides, presumably the growing points of the wall.

In *S. aureus* extensive interpeptide crosslinking occurs, so that after cleavage of the polysaccharide virtually all of the peptide remains as a high-molecular-weight substance.[30] The nature of this peptide bridge varies among bacterial species. In *S. aureus* it contains an average of five glycine residues, attached at one end to the ϵ-amino groups of lysine.[24, 29, 30] At the other end it could be attached either to the α-carboxyl group of glutamic acid (since the γ-carboxyl group is in the tetrapeptide chain) or to the C-terminal D-alanine of the tetrapeptide.

However, it has now been shown that the α-carboxyl group of glutamic acid is an amide, thus excluding this point of attachment for the glycine bridge. It was assumed earlier[13] that the ammonia in the glycopeptide of *S. aureus* was derived from degradation of the amino sugars during hydrolysis. However, analysis of a polypeptide[30] (containing tetrapeptides with N-terminal L-alanine, linked by pentaglycine bridges) and of a pentapeptide[31] (containing the tetrapeptide plus one glycine residue) obtained during studies of wall structure indicated that 1 mole of ammonia was present in these carbohydrate-free fragments. In order to establish the location of the ammonia residue, Edman degradation was carried out[32] (Table 1). After the first cycle of degradation of the polypeptide, its N-terminal alanine was removed and N-terminal glutamic acid appeared. After the second cycle of Edman degradation, ammonia was liberated and the N-terminal groups disappeared. DL-isoglutamine, employed as a standard, similarly yielded ammonia in this reaction. The monoglycine-substituted tetrapeptide fragment, obtained from *S. aureus* cell walls after degradation with a lytic peptidase[33] also contained one N-terminal L-alanine residue per glutamic acid, and also yielded first N-terminal glutamic acid and then ammonia on Edman degradation. Degradation of a carbohydrate-free peptide obtained from *Arthrobacter crystallopoietes* has similarly shown that D-isoglutamine is the second amino acid in this peptide sequence, while in *M. lysodeikticus* the α-carboxyl group of glutamic acid in the tetrapeptide is substituted by a glycine residue with a free carboxyl group.[31]

In addition, linkage of the amino end of the glycine chain to the carboxyl group of the terminal D-alanine in the tetrapeptide has been directly demonstrated. Both the L_{11} enzyme from *Flavobacterium*[34] and an endopeptidase from *Streptomyces albus*[29] are glycine bridge-splitting enzymes. These enzymes open these bridges by liberation of N-terminal glycine plus both C-terminal glycine and C-terminal D-alanine. In the best experiment 75 per cent of the bridges were opened with formation of C-terminal D-alanine.

TABLE 1

LIBERATION OF AMMONIA FROM THE POLYPEPTIDE DURING EDMAN DEGRADATION

Number of degradations	Ammonia		N-Terminal Amino Acids	
	Total	Free	Glutamic	Alanine
0	107	0	9	91
1	—	13	92	0
2	76	55	5	0

Data are expressed as moles/100 moles total glutamic acid. The polypeptide and its degradation products also contained small amounts of N-terminal glycine and lysine (5–10 residues). The procedure for the first degradation cycle was a micromodification of that described by Konigsberg and Hill,[32] but ring closure in the second cycle was performed in 4 *N* HCl under conditions such that the hydrolysis of isoglutamine was negligible. A similar yield (70%) of ammonia was obtained from isoglutamine after this degradation sequence. For methods, see Table 2.

Biosynthesis of the Cell Wall.—The utilization of UDP-MurNAc-pentapeptide and UDP-GlcNAc for wall biosynthesis involves the participation of a membrane-bound phospholipid.[17]

In this process, GlcNAc-MurNAc(-pentapeptide)-P-phospholipid is an intermediate. Then a pentaglycine chain is added to the ϵ-amino group of lysine in the pentapeptide to form GlcNAc-MurNAc(-pentapeptide-pentaglycine)-P-phospholipid.[19] These lipid intermediates presumably serve as a means by which intracellular nucleotide precursors (to which the cell membrane is impermeable) are utilized for the synthesis of an essentially extracellular product, the cell wall. A linear polymer of uncrosslinked alternating GlcNAc and MurNAc-decapeptide units is formed from the lipid intermediate; it still retains both of the D-alanine residues in UDP-MurNAc-pentapeptide.[17, 19] The utilization of the lipid intermediates for glycopeptide synthesis is inhibited by ristocetin, vancomycin, and bacitracin.[16, 17] but neither their formation nor utilization is inhibited by penicillin. Thus, the locus of action of penicillin must lie in some subsequent reaction.

At least three additional reactions must occur in synthesis of the glycopeptide of *S. aureus:* O-acetylation of some of the MurNAc residues,[30] amidation of the α-carboxyl group of glutamic acid, and closure of the glycine bridges. It is postulated that the loss of the terminal D-alanine residue is directly coupled to bridge closure and that the energy of the D-alanyl-D-alanine linkage is conserved in a transpeptidation in which the bridge is closed. This hypothesis is attractive because bridge closure must occur on the cell wall at the outside of the cell membrane, and at this extracellular site ATP is almost certainly not available as an energy source for synthetic reactions. The hypothesis formulated by Wise and Park[22] makes similar points. On the other hand, the O-acetylation and amidation steps probably require the participation of ATP as an energy source, and should therefore take place before membrane transport and transfer to glycopeptide. Indeed, by increasing the lipophilic nature of the glycopeptide unit, they would presumably facilitate this transport.

Molecular Models of Penicillin and of Acyl-D-alanyl-D-alanine.—Penicillin is in essence an acylated cyclic dipeptide of L-cysteine and D-valine.[1, 35] It can be viewed as an analog of the acylated D-alanyl-D-alanine in the linear glycopeptide. The occurrence of D-isoglutamine in the cell wall and in the nascent glycopeptide units (see below) makes it unlikely that penicillin with its free carboxyl group is an analog of L-alanyl-D-glutamic acid in the peptide chain, as suggested by Wise and Park,[22] and no evidence has been presented to support an earlier idea that penicillin is a structural analog of acetylmuramic acid.[36] In fact, the terminal D-alanine contains the only free carboxyl group in the nascent glycopeptide which might resemble that in penicillin.

The atoms of the peptide chain in penicillin are fixed in position by the ring system. One of the possible conformations of the peptide chain of D-alanyl-D-alanine is almost identical to that of penicillin (Fig. 1) (in spite of the fact that one of the carbon atoms of penicillin has the L-configuration). In the conformation photographed, the methyl group of the D-alanyl residue has a proton as its analog in the penicillin molecule. The *absence* of a methyl group in this position would presumably not inhibit the binding of penicillin to an enzyme whose binding site normally accommodated this group. This situation is analogous to and was in fact suggested by the recently described relationships between L-alanine, D-alanine, and D-cycloserine as substrates and inhibitor for alanine racemase.[37] Both L- and D-alanine can assume the conformation of D-cycloserine, and all three are

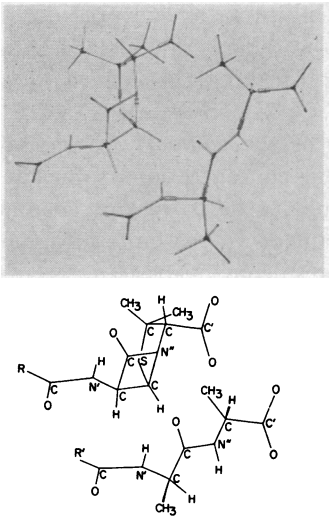

FIG. 1.—Photograph of Dreiding stereomodels of a substituted 6-aminopenicillanic acid (penicillin) and a substituted D-alanyl-D-alanine. The distances N′ to N″ (3.3 Å) and N″ to C′ (2.5 Å) in the two models are identical, while the distances N′ to C′ (5.4 Å, penicillin, and 5.7 Å, D-alanyl-D-alanine) are quite similar. The carbon atom common to the two rings and the sulfur atom of the penicillin nucleus have no analog in the D-alanyl-D-alanine structure. They serve to fix the configuration, and presumably they would not affect the approach of an enzyme molecule to the top (as photographed) of the molecules. The major difference between the models depends on the configuration about N″, determined in penicillin by the ring structures, and in D-alanyl-D-alanine by the double-bond character of the CO—N bond. It seems likely that a transition state during the scission of this bond would have approximately single-bonded character with a nonplanar configuration about N″. Such a model (not illustrated) can be made to fit the appropriate parts of the penicillin skeleton very closely indeed, and the N′ to C′ distances are then identical.

bound to alanine racemase. L-cycloserine, although it belongs to the same configurational series as L-alanine, cannot assume the same conformation and hence it is not an inhibitor of alanine racemase. A similar conformational situation has been described for the various substrates of glutamine synthetase.[38]

It is therefore postulated that penicillin has the conformation in which the D-alanyl-D-alanine end of the acetylmuramyl-pentapeptide fragment is fixed to the substrate binding site of the transpeptidase. It is further postulated that the transpeptidase normally catalyzes a reaction in which the D-alanyl-D-alanine bond in the substrate is cleaved, presumably with conservation of the bond energy as a substituted D-alanyl-enzyme intermediate. The bridge would then be closed by transfer of the D-alanyl residue to the amino end of the glycine chain (Fig. 2).

In this view the highly reactive amide bond of the β-lactam ring of penicillin is the equivalent of the peptide bond in D-alanyl-D-alanine. When fixed to the transpeptidase, a very facile acylation of the transfer site would occur with opening of the β-lactam ring, forming a penicilloyl enzyme, thus inactivating the transpeptidase. Penicillin is in fact irreversibly bound to a "penicillin-binding component" of bacterial cells, which is probably the protein component of a lipoprotein complex located at the outside of the cell membrane,[39, 40] presumably the transpeptidase. An interesting extension of the hypothesis is the possibility that penicillinase is a modified transpeptidase, no longer bound to the lipid membrane, and in which the penicilloyl enzyme is readily hydrolyzed by water. In fact, the sites of binding of penicillin for killing and for induction of penicillinase are closely related.

Accumulation of Uncrosslinked Nascent Glycopeptide Fragments in the Presence of Penicillin.—Penicillin G at concentrations of 0.08, 0.15, 0.3, and 1.0 μg/ml was added to early log-phase cultures (500 ml) of *S. aureus* strain Copenhagen. After preincubation for 10–40 min, C^{14}-glycine was added. Thirty min-

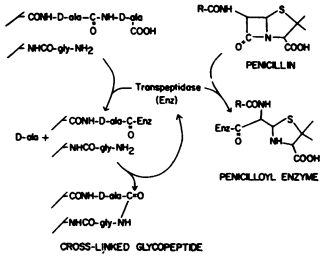

FIG. 2.—Proposed transpeptidation sequence and its inhibition by penicillin.

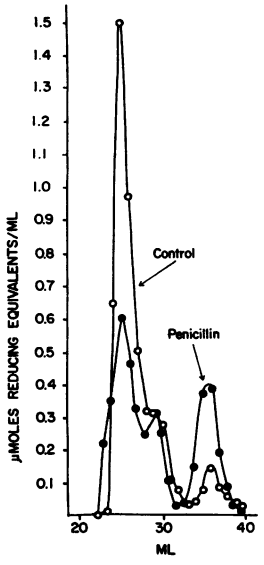

FIG. 3.—Accumulation of uncrosslinked glycopeptide fragments induced by penicillin (0.08 µg/ml). For details, see text. The void volume of this Sephadex G-25 column is 24 ml and salts are eluted at 50 ml. Reducing power measures total material since the polysaccharide has been completely fragmented into disaccharides. The plot of incorporated C^{14}-glycine was similar with peaks in identical positions.

utes later, when the penicillin-treated cultures departed from exponential growth, the cells were collected and cell walls were prepared after breaking the cells with glass beads.[30] Incorporation of C^{14}-glycine was 49, 35, 31, and 23 per cent, respectively, of the control. It was hoped that at these low penicillin concentrations sufficient defective wall synthesis would occur to permit accumulation of a measurable amount of uncrosslinked fragments. In *Proteus mirabilis* where penicillin-treated organisms continue to grow and multiply as L-forms with defective cell walls,[20, 21] obviously extensive wall synthesis continues in the absence of a normal crosslinking reaction.

The cell walls were then lysed with an endoacetylmuramidase.[41, 42] The teichoic acid-glycopeptide complex was absorbed on Ecteola-cellulose, and the glycopeptide (disaccharides linked by polypeptide) in the water effluent of this column was filtered on a column of Sephadex G-25 (Fig. 3). In the control glycopeptide, 70 per cent of the material was eluted in a peak which was excluded from the gel. About 7 per cent was present as a low-molecular-weight fragment which contained 9 per cent of the newly formed glycopeptide as measured by C^{14}-incorporation. In the penicillin-treated cells, on the other hand, 40, 57, 60, and 51 per cent, respectively, of the incorporated C^{14}-glycine occurred in this peak. In absolute amount, it contained 28, 27, 20, and 15 per cent, respectively, of the total reducing material. It appears that at low penicillin concentrations a weakened cell wall may be formed containing large amounts of nascent uncrosslinked units. At higher penicillin concentrations little crosslinked glycopeptide was formed and the accumulation of the nascent units was then suppressed; wall synthesis ceased. The formation of a weakened wall at low concentrations may explain the paradoxical observation that the killing rate can be greater at low than at high penicillin concentrations.[43] By contrast, with either bacitracin or vancomycin (Fig. 4) much of the low-molecular-weight fragment disappeared. These two antibacterial agents apparently do not prevent the crosslinking of nascent glycopeptide, but interfere with its formation.

Analyses of the low-molecular-weight fragments from penicillin-treated and control cells (Table 2) indicated that both were GlcNAc-MurNAc(-pentapeptide (amide)-pentaglycine), containing two D-alanine residues, one of which was C-terminal, and one N-terminal glycine. In order to obtain material similar to this repeating "monomer" of the glycopeptide for comparison, the soluble glycopeptide from *S. aureus* was treated with the L_{11} enzyme, a peptidase which hydrolyzes the glycine bridges.[31, 34] The reaction was stopped when 65 per cent of the bridges had been hydrolyzed in order to obtain "oligomers" of the glycopeptide, as well as "monomer." Filtration on Sephadex G-25 (Fig. 4) revealed the expected distribution of fragments, and analyses confirmed that the lowest-molecular-weight fraction

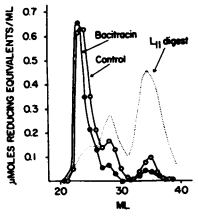

FIG. 4.—Effect of bacitracin (75 μg/ml) on distribution of glycopeptide fragments. The method was essentially the same as described for penicillin. Vancomycin (7.5 μg/ml) yielded similar results. Note the marked decrease in the monomer eluted at 35 ml. The total amount of C^{14}-glycine incorporated in the presence of bacitracin was 31% of the control, and incorporation into the monomer was 55% of the control. On the other hand, the glycopeptide from vancomycin-treated cells contained no monomer and the incorporation of C^{14}-glycine was only 2% of the control. These data indicate that the block in formation of the monomer induced by bacitracin was incomplete, while that induced by vancomycin was complete, just as had been observed in studies of inhibition of the polymerization reaction *in vitro*.[17] The dotted line represents fractionation on the same column of the products of hydrolysis of the high-molecular-weight glycopeptide by the L_{11} enzyme (kindly given by Dr. K. Kato).

was a monomer containing GlcNAc-MurNAc(-tetrapeptide(amide)-pentaglycine) differing from the material isolated from cell walls of normal or penicillin-treated cells in lacking one D-alanine residue. On a column of Bio-Gel P2, a molecular weight for these fragments in the range of 1100–1500 was estimated from the elution volumes. Their calculated molecular weights are 1210 and 1280. Paper chromatography of these fragments in isobutyric acid: 1 *N* NH_4OH (5:3) showed that each contained two components. The mobilities of the two fragments from the L_{11} hydrolysate (R_f = 0.52 and 0.65) and of those isolated from the penicillin-treated cells (R_f = 0.54 and 0.67) were extremely similar. Presumably the faster-moving component in each case is the O-acetylated monomer.[29, 30] The presence of an amide in the nascent glycopeptide indicates that the amidation reaction precedes bridge closure and that it is not inhibited by penicillin.

When cell walls were prepared by extraction of cells with hot trichloroacetic acid, considerable breakage of glycine bridges occurred leading to artificial production of uncrosslinked fragments; the procedure was therefore not used further. Using this method, however, Wise and Park[22] showed a significant increase in the incorporation of labeled alanine into the glycopeptide in the presence of penicillin, and a significant increase in newly incorporated N-terminal glycine over the high background due to broken glycine bridges.

Since it is proposed that penicillin acylates the transpeptidase in a manner analogous to its acylation by D-alanine in the glycopeptide, it was also possible that the penicilloyl-transpeptidase could transfer penicilloyl to the end of a glycine chain in a manner analogous to bridge closure, thereby acting as a "chain terminator." Cell walls were therefore isolated from cells incubated with C^{14}-penicillin G. They,

TABLE 2

ANALYSES OF REPRESENTATIVE GLYCOPEPTIDE FRACTIONS

Fraction	Disac	Gly	Lys	Ala	L-ala	D-ala	C-terminal D-ala	N-terminal gly	Amide
Polymer (penicillin)	102	480	94	201	79	79	—	8	81
Monomer (penicillin)	104	489	104	286	100	197	105	84	78
Monomer (control)	104	480	109	283	88	164	56	65	116

Data are expressed as moles per 100 moles of glutamic acid. Polymer is material eluted from Sephadex G-25 at 24 ml and monomer is the material eluted at 35 ml (Fig. 3). The penicillin concentration was 0.08 μg/ml. Similar analyses were obtained for the corresponding peaks of all preparations. Disaccharide was measured by total hexosamine, reducing power, and 30-min Morgan-Elson reactions, total amino acids by quantitative thin-layer chromatography of dinitrophenyl derivatives of hydrolysates, D-, and L-alanine by specific enzymatic procedures, C-terminal amino acids after hydrazinolysis, N-terminal amino acids after dinitrophenylation, and amide ammonia by the increase in free ammonia[44] after hydrolysis. Methods are described in refs. 29, 30, and 45. All of the C-terminal amino acid was D-ala, and all of the N-terminal amino acid was glycine.

as well as the monomer and oligomers obtained from them, contained only a trace of radioactivity. No significant transfer of penicilloyl groups to glycine in the glycopeptide could have occurred.

The hypothesis explains the requirement for a free carboxyl group and a substituted 6-amino group for penicillin action, as well as the fact that expansion of the thiazolidine ring (as in cephalosporins) does not destroy activity as long as a free carboxyl group in the same position is retained. However, the presence of the β-lactam ring is essential for activity. The present studies suggest additional modifications of penicillin which might result in even more effective antibacterial agents. In this hypothesis acyl substituents (phenylacetic acid in penicillin G) on 6-aminopenicillanic acid (or 7-aminocephalosporanic acid) may be regarded as analogs of the amino acids preceding D-alanyl-D-alanine in the acetylmuramyl-pentapeptide. Thus, addition of lysine, substituted lysines, derivatives of diaminopimelic acid or of other dibasic amino acids found in cell walls, or small peptides related to the cell wall tetrapeptides should result in an even closer resemblance to the linear glycopeptide substrate; possibly lipophilic substituents are necessary to permit penetration to the transpeptidation site. Similarly, a 6-methyl penicillin (or 7-methyl cephalosporin) would bear a methyl group in the same position as is found in the D-alanyl residue and this might enhance its effectiveness.

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*INHIBITION OF DNA SYNTHESIS IN EHRLICH ASCITES CELLS BY ACTINOMYCIN D, II. THE PRESYNTHETIC BLOCK IN THE CELL CYCLE**

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The interval between midpoint of mitosis in the parent cell and midpoint of the subsequent mitosis in the daughter cell is called the cell cycle[1] and is schematically represented in Figure 1. After completion of mitosis, the cell enters a presynthetic phase, the G_1 phase. It then synthesizes DNA (S phase), and when DNA synthesis ceases, it goes through a premitotic phase G_2 which is followed by a new mitosis, M.

In a previous paper,[2] we have shown that small doses of actinomycin D (0.016 μg/gm body weight) produce two distinct effects in Ehrlich ascites cells growing in the peritoneal cavity of mice: (1) a prompt 50 per cent inhibition of RNA synthesis; and (2) a delayed 50 per cent inhibition of DNA synthesis. The present communication shows that the delayed inhibition of DNA synthesis is due to a presynthetic block in the G_1 phase of the cell cycle and that the RNA mostly affected by these small doses of actinomycin D is ribosomal RNA. The over-all results then indicate that there is an actinomycin D-sensitive step in the G_1 phase of the Ehrlich ascites cells and that inhibition of this step prevents the entrance of tumor cells into the S phase of the cell cycle.

Methods and Materials.—These have been described in detail in the previous paper.[2] All experiments were performed on 5-month-old Strong A female mice on the 6th day after an intraperitoneal injection of Ehrlich ascites cells.

Autoradiography: Smears of Ehrlich ascites cells were fixed in methanol and autoradiographs